Chromosomal Rearrangements During Turtle Evolution Altered the Synteny of Genes Involved in Vertebrate Sex Determination

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Abstracts

Sex-determining mechanisms (SDMs) set the individual's sexual fate by its genotype (GSD) or environmental factors like temperature (TSD), as in turtles where the GSD "trigger" remains unknown. SDMs co-evolve with turtle chromosome number, perhaps because fusions/fissions alter the relative-position/regulation of sexual development genes. Here, we map ten such genes via fluorescent-*in-situ*-hybridization (FISH) onto metaphase chromosomes in TSD and GSD turtles for the first time. Results uncovered intra-chromosomal rearrangements involving three genes across SDMs, and a chromosomal fusion linking two genes in one chromosome in a TSD turtle that locate to two chromosomes in all others. Notably, *Sf1* and its repressor *Foxl2* map to *Apalone spinifera*'s ZW but to a macro- (*Foxl2*) and a micro-autosome (*Sf1*) in other turtles potentially inducing SDM evolution. However, our phylogenetically-informed analysis refutes *Foxl2* (but not *Sf1*) as *Apalone*'s master sex-determining gene. The absence of common TSD/GSD rearrangements underscores the independent evolutionary trajectories of turtle SDMs.

Introduction

Primary sex determination is the biological process that directs an individual's sexual fate toward the formation of ovaries or testis. Notably, while sexual reproduction is one of the most taxonomically conserved traits, the mechanisms of sex determination (SDMs) that commit the developing embryo to a phenotypic sex are incredibly diverse and evolve frequently. Even in closely-related species, there can be differences in both primary signal and downstream genetic pathways (Bachtrog et al. 2014; Haag and Doty 2005). Across the tree of life, SDMs range from those directed by sex-specific genotypes (genotypic sex determination, GSD) to those directed by environmental factors such as temperature (temperature-dependent sex determination, TSD); the latter being commonly found in reptiles (Bachtrog et al. 2014). The

vast diversity and complexity of SDM harbored within vertebrates still puzzles researchers and our understanding of how SDMs evolves remains incomplete.

Most turtles exhibit the ancestral TSD from which GSD evolved independently multiple times, sometimes as male-heterogametic (XX/XY) and other times as female-heterogametic (ZZ/ZW) sex chromosome systems (Bachtrog et al. 2014; Sabath et al. 2016; Valenzuela and Adams 2011). Phylogenetic analyses vary in their estimate of the number of evolutionary transitions among sex-determining modes that are reconstructed in turtles (Sabath et al. 2016; Valenzuela and Adams 2011). Recent molecular evidence (Literman et al. 2017) supports the scenario that five transitions from TSD to GSD and two reversals back to TSD from GSD occurred during turtle evolution (Valenzuela and Adams 2011). Yet, little is known about the molecular basis and consequences of the transitions between GSD and TSD in closely related taxa, especially in turtles, a group where no GSD trigger has been identified. However, accelerated rates of change in chromosome number are associated with changes in sex-determining mechanism in turtles (Valenzuela and Adams 2011). Potential explanations for this association are that chromosomal rearrangements underlying diploid number evolution (change in chromosome number) may trigger SDM transitions, or alternatively, that SDM transitions may alter molecular processes that in turn, render the genome unstable and lead to chromosomal rearrangements (Valenzuela and Adams 2011). Here we examine the first of these hypotheses.

Chromosomal rearrangements include fissions, fusions, deletions, duplications, inversions, and translocations, and they are known to perturb the organization and function of elements whose relative position are affected (Harewood and Fraser 2014). For instance, chromosomal rearrangements can impact gene expression (Harewood and Fraser 2014), either by direct physical disruption, by changing regulatory elements, or by creating positional effects, as in the case of many Mendelian disorders in humans, some of which are associated with sex determination (Harewood et al. 2012). Examples include the translocation breakpoint of *Sox9* which is associated with male-to-female sex reversal in XY patients (Velagaleti et al. 2005), inversions linked to the aromatase (*CYP19*) excess syndrome that place this gene under the control of the promoter of other expressed genes (Demura et al. 2007), and the translocation of *Sry* from the human Y to the X chromosome in XX hermaphrodite patients (Margarit et al.

2000). Whether chromosomal rearrangements alter the relative location of genes and syntenic groups of the gene regulatory network of sexual development in turtles (Czerwinski et al. 2016; Radhakrishnan et al. 2017; Valenzuela 2008a) remains unknown. We address this question here.

Turtles are reported as possessing highly conserved karyotypes compared to other reptiles such as lizards and snakes (Bickham 1981; Olmo 2008), yet turtles vary substantially in chromosome numbers (from 2N=26-68). Turtle karyotypes are characterized by two chromosomal components, macro- and micro-chromosomes (Olmo 2008). Importantly, changes in turtle diploid number involve mostly variation in the number of micro-chromosomes (Montiel et al. 2016a; Olmo 2008). In general, small micro-chromosomes are defined as those that are nearly indistinguishable from each other by shape and centromere position due to their small size.

Despite the variety of vertebrate SDMs, with few exceptions, virtually the same cascade of genes underlying gonadal development in the sex determination network are common to many vertebrates, including reptiles such as GSD and TSD turtles (Czerwinski et al. 2016; Radhakrishnan et al. 2017; Valenzuela 2008a; Yatsu et al. 2016). Here we examine the relative chromosomal position of a set of genes in the regulatory network of sex determination/differentiation, including genes implicated in testicular development (*Dmrt1*, *Fgf9*, and *Sox9*) (Ferguson-Smith 2007), ovarian development (*Dax1*, *Foxl2* and *Rspo1*), and genes important in both pathways such as some that regulate general gonadogenesis prior to sexual commitment (*Fhl2*, *Gata4*, *Sf1*, and *Wt1*) (Valenzuela 2008a) (Table 1).

These target genes were selected because turtle bacterial artificial chromosome (BAC) clones containing them were available from a BAC library of painted turtle (*Chrysemys picta*) (library VMRC CHY3 produced by the Joint Genome Institute) and they had been previously mapped in the chromosomes of painted turtle (Table 2) (Badenhorst et al. 2015). BAC clones contain DNA fragments of genomic sequences between 100-200 Kbp in size, which can be used as probes for *in situ* hybridization onto chromosomes to detect their physical locations (Janes et al. 2011).

We used BAC-based fluorescent-*in-situ*-hybridization (BAC-FISH) to detect changes in chromosomal positions that might occur when the order of genes is modified due to chromosomal rearrangements. We examined the patterns of synteny of the target genes among 12 focal species given the current hypothesis of the phylogenetic relationships of the studied turtles (as used in Valenzuela and Adams 2011) (Fig. 1A), such that changes from the common ancestor could be detected. With these data, we tested whether evolutionary changes in the synteny of these focal genes across six TSD and six GSD taxa help identify genomic regions whose evolution may be associated with transitions in turtle sex determination (Fig. 1A). Our results represent the first partial physical mapping data for some of these turtle species. Our data uncovered intra-chromosomal rearrangements that altered the relative position of some genes within a single chromosome in several taxa, as well as fusion/fission events that explain the localization of two genes in two chromosomes in some taxa and their co-localization in single chromosome in other species. Additionally, BAC-FISH data revealed sex-linkage of some of these genes in a turtle with evolutionarily derived GSD that render them potential candidate sex-determining genes for future functional testing.

Materials and Methods

Cell culture and chromosomes preparation

Metaphase chromosome preparations were obtained from cell cultures following standard procedures as described previously (Badenhorst et al. 2015; Montiel et al. 2016a; Montiel et al. 2016b) for 12 turtle species with either TSD or GSD ranging in diploid number 2N = 28-66 (Fig. 1A): *Chrysemys picta* (CPI), *Trachemys scripta* (TSC), *Chelydra serpentina* (CSE), *Glyptemys insculpta* (GIN), *Staurotypus triporcatus* (STR), *Sternotherus odoratus* (SOD), *Apalone spinifera* (ASP), *Pelodiscus sinensis* (PSI), *Podocnemis unifilis* (PUN), *Pelomedusa subrufa* (PSU), *Chelodina oblonga* (COB), and *Emydura macquarii* (EMA). Hereafter we refer to these species by their three-letter acronym or genus name. Briefly, primary fibroblast cell cultures for cytogenetic analyses were established from turtle tissues digested with collagenase (Gibco) and

cultured using a medium composed of 1:1 RPMI 1640:Leibowitz media (Gibco) supplemented with 15% fetal bovine serum (One Shot, Gibco), 2 mM L-glutamine (Gibco), and 1% antibiotic-antimycotic solution (Gibco). Cell cultures were incubated at 30°C without CO_2 supplementation. Four hours prior to harvesting, 10 µg/ml colcemid (KaryoMAX®, Gibco) was added to the cultures. Metaphase chromosomes were harvested after hypotonic exposure and fixed in 3:1 methanol:acetic acid. Cell suspensions were dropped onto glass slides and air dried.

BAC clone fluorescent-in-situ-hybridization (BAC-FISH) mapping

The previously mapped BAC clones from the *Chrysemys* CHY3 BAC library (Badenhorst et al. 2015) examined here either carry genes of interest (*Dax1*, *Dmrt1*, *Fgf9*, *Sf1*, *Sox9*, and *Wt1*), or contain sequences of genome scaffolds from the *Chrysemys* genome assembly (Badenhorst et al. 2015; Shaffer et al. 2013) that span target genes (*Fhl2*, *Foxl2*, *Gata4*, and *Rspo1*) (Table 2). A schematic summary of the positions of genes of interest and each BAC are presented in Fig. 2. BAC sequences were obtained from either NCBI (Shaffer et al. 2013), by direct 454 sequencing of entire BACs, or by Sanger sequencing of PCR products amplified using gene-specific primers. BAC DNA (~1 µg) was extracted and labeled by standard nick-translation (Abbott Molecular) using either biotin-16-dUTP or digoxigenin-11-dUTP (Roche) and co-precipitated with human cot-1 DNA and *Chrysemys* cot-1 DNA to suppress repetitive DNA sequences. BAC-FISH was carried out using these labeled BAC probes, as described by Montiel et al. (2016a, 2016b). Briefly, chromosome slides were incubated at 65°C for 2 hours, denatured 1 min 45 sec at 70°C in 70% formamide/2× SSC, dehydrated through an ethanol series and air dried. A 15-µL mixture containing BAC probes was hybridized to each slide in a humid chamber (wet paper in air-tight container) at 37°C for 3 nights (for more closely related species) or 5 nights (for more distantly related species) (Fig. 1A). Slides were washed post-hybridization twice, first in 0.4× Saline-Sodium Citrate (SSC)/0.3% Tween-20 for 2 min at 60°C, and then in 2× SSC/0.1% Tween-20 for 1 min at room temperature. A 200-µl solution of 4XT/relevant-antibody (fluorescein-conjugated avidin or anti-digoxigenin-rhodamine) was used for fluorochrome detection at 37°C for 1 hour. Slides were subsequently washed three times in 4XT (4× SSC/0.05% Tween-20) at 37°C and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Slides were then mounted using an antifade solution (Vectashield). The FISH signal and hybridization pattern determined the BAC

clones' relative position within chromosomes, and chromosomes were identified according to their morphology, size, shape, and DAPI-banding (Fig. 3) as previously described (Montiel et al. 2016a). A minimum of 10 complete metaphase spreads were examined and analyzed for each gene per specimen. Images were taken with a Leica DFC365 FX camera attached to an Olympus BX41 fluorescent microscope and analyzed using CytoVision® cytogenetic analysis system. Chromosome homology across species (Fig. 1A) was determined by extensive BAC-FISH data (Montiel et al., unpublished) and corroborated by whole-chromosome painting data collected for a parallel study (Valenzuela et al., unpublished). We note that for four of our target genes we used BACs whose sequence mapped to *Chrysemys*, the painted turtle genome scaffold containing the gene of interest (*Fhl2*, *Foxl2*, *Gata4*, *and Rspo1*) (Table 2). In order to assess the likelihood that these BACs are also good proxies for the position of those genes in turtles other than *Chrysemys*, we investigated the published genome assemblies of several turtles [*Malaclemys terrapin* (Pop et al., unpublished), *Chelonia mydas (Wang et al. 2013)*, *Gopherus agassizi (Tollis et al. 2017)*, *Pelodiscus sinensis (Wang et al. 2013)*], plus the genome assemblies of *Staurotypus triporcatus* and *Apalone spinifera* we obtained ourselves (Valenzuela et al., unpublished data). This approach corroborated that the sequence of these BACs also mapped to scaffolds containing these four genes of interest at least two other genomes, being the distance between them consistent across species ~~(Table S1)~~, or alternatively, when the genomic distance in between was too long to be covered by a single scaffold in the fragmentary published genome assemblies, both BAC and gene can be found in scaffolds that also show homology with the flanking regions of both the BAC and the gene, thus increasing our confidence that our FISH data informed the chromosomal location of these four target genes across turtles.

Results

Here, we examined the chromosomal location of a set of 10 genes from the regulatory network of sexual development (*Dax1*, *Fhl2*, *Fgf9*, *Sox9*, *Wt1*, *Dmrt1*, *Rspo1*, *Sf1*, *Foxl2*, and

Gata4) and uncovered the following intra-chromosomal inversions, gene translocation, and a chromosomal fusion/fission event that occurred during turtle evolution (full FISH results are presented in Fig. 4). First, BAC clone probes for genes *Fhl2*, *Dax1*, and *Fgf9* (Table 2), painted onto the largest chromosome—in all turtles (i.e. chromosome 1, which is homologous across taxa), with the exception of *Emydura* (a GSD turtle), where they painted onto the third largest chromosome (EMA3) (Fig. 1A and Fig. 1B). Based on the FISH data of multiple BAC clones that mapped to CPI1, EMA3 appears to be homologous to the long arm of CPI1 (Montiel *et al.*, unpublished). Although these three genes painted in a single chromosome in all cases, our FISH results did uncover intra-chromosomal rearrangements that alter their relative position in several taxa (Fig. 1B). Namely, these three genes localized in the long arm of chromosome 1 in the same order (*Dax1-Fhl2-Fgf9* from the centromere to the telomere of the q-arm) across *Trachemys*, *Glyptemys*, *Chelydra*, *Staurotypus*, *Sternotherus*, and *Chelodina* (some of which exhibit TSD and some GSD, Fig. 1A), but displayed a different gene order in *Chrysemys*, *Apalone*, *Pelodiscus*, and *Pelomedusa* (*Fhl2-Dax1-Fgf9*) (also spanning TSD and GSD taxa), and showed a unique pattern in EMA3 (*Fhl2-Fgf9-Dax1*).

Additionally, FISH data uncovered a likely chromosomal fusion or translocation event involving *Sf1* and *Rspo1* in *Pelomedusa*, as they co-localize in a single chromosome in *Pelomedusa* but in two different chromosomes in all other taxa with contrasting sex determination (Fig. 3C, 2D, and 2E). Notably, our results also revealed that BAC clones containing *Sf1* and *Foxl2* mapped on the sex chromosomes of *Apalone* (Fig. 3G and 3H), whereas in other turtles (including *Apalone*'s close relative *Pelodiscus* with which it shares a homologous ZZ/ZW system), *Sf1* is located in a micro-autosome (Fig. 3E).

Discussion

We examined the chromosomal location of ten genes (*Dax1*, *Fhl2*, *Fgf9*, *Sox9*, *Wt1*, *Dmrt1*, *Rspo1*, *Sf1*, *Foxl2*, and *Gata4*) known to be involved in gonadogenesis in vertebrates, across 12 focal turtle species in a phylogenetic context, to help elucidate the evolutionary process associated with turnovers in sex determination. Our results uncovered genomic

rearrangements in turtles that altered the relative position of gene regulators of sexual development and contribute to the search for candidate sex-determining genes in GSD turtles.

Gene translocation of Sf1 and Foxl2 is tied to the derived sex chromosome system in Apalone

Genes harbored in the sex chromosomes are of particular interest due to their potential unique roles in sex determination and sexual dimorphism (Pask and Graves 1999). Sex chromosomes originate from autosomes when a gene assumes the sex-determining role, either by recruitment of a new sex-limited master-switch trigger or by gene dosage effects on gonadal differentiation (Bachtrog et al. 2014). Thus, the Y or W chromosomes can provide primary sex determination signals that commit the embryo to its developmental sexual fate (Bachtrog et al. 2014). As such, genes linked to sex chromosomes are prime candidates for a master role in sex determination which could trigger the downstream genes of the sexual development network, whereas autosomal genes may contribute to gonadal formation, but may be ruled out for the master trigger function.

Such master triggers have been identified in Therian mammals, birds, and fish among other vertebrates [reviewed in (Bachtrog et al. 2014)], but not in turtles. However, several genes involved in sexual development were reported to reside in the sex chromosomes of GSD turtles, such as *Wt1* in *Glyptemys* (Montiel et al. 2016b) and *Dmrt1* in *Staurotypus* (Kawagoshi et al. 2014). Both are XX/XY species. Here we found that *Sf1* and *Foxl2* map to *Apalone's* sex chromosomes, which at first glance would make them both candidate sex-determining genes in this GSD turtle with heteromorphic sex chromosomes (Badenhorst et al. 2013) but would require an unlikely scenario of evolutionary events as described below. *Foxl2* is a transcription factor essential for the maintenance of the ovaries which suppresses testicular differentiation mainly through repression of *Sox9 (Uhlenhaut et al. 2009)*. *Foxl2* is upregulated at female-producing temperatures in TSD reptiles including several turtles and alligator (Janes et al. 2013; Radhakrishnan et al. 2017; Rhen et al. 2007; Shoemaker-Daly et al. 2010), and it displays relic thermosensitive transcription in *Apalone* (Radahnkrishnan et al 2017). Notably, our mapping data in the context of the phylogenetic relationships of the species examined here (Fig. 1A)

indicate that *Foxl2* ancestral location was in a macro-autosome [chromosome 9 based on decreasing size (Montiel et al. 2016a)], from which it translocated to the micro- sex chromosomes in *Apalone* (Badenhorst et al. 2013). Whether the translocation occurred after *Apalone*'s split from *Pelodiscus*, with whom it shares a homologous ZZ/ZW sex chromosome system (Badenhorst et al. 2013), or at the split of the Trionychidae family lineage warrants further investigation, as we were not successful in mapping *Foxl2* in *Pelodiscus*. Alternatively, our failure to map *Foxl2* in *Pelodiscus* may be indicative of the divergence of the genomic region encompassed by this BAC clone since the split of *Apalone* and *Pelodiscus* ~95Mya. This hypothesis is supported by our bioinformatics examination of the available turtle genome assemblies which revealed the presence of *Sf1* and *Foxl2* in a single genomic scaffold in *Apalone* but not in *Pelodiscus* (results no shown). Nonetheless, if the sex-linkage of *Foxl2* in *Apalone* is secondary, it would imply that for *Foxl2* to be the master trigger of sex-determination, it would have had to replace the original sex-determining gene that must have arisen at the origin of sex chromosomes in the family of softshell turtles (Trionychidae). While plausible, this is an unlikely scenario. On the other hand, *Sf1* is a nuclear orphan receptor required for the formation of the adrenal gland and gonads, as well as for the expression of steroidogenic enzymes therein (Morohashi 1999; Parker and Schimmer 1997). The expression of *Sf1* is evolutionarily labile among vertebrates, and depending on the taxon, it displays expression patterns compatible with a role in testis development, ovarian development, or a general role in gonadal development (Valenzuela et al. 2013). *Sf1* was previously proposed as a key GSD regulator in the *Apalone* genus based on the lack of differential gene expression detected in *A. mutica* in contrast to its thermosensitive transcription in the TSD painted turtle, *Chrysemys (Valenzuela 2008a)*. Similar results were detected in *A. spinifera (Radhakrishnan et al. 2017)*. *Sf1* lies directly downstream from *Wt1* and *Dax1* in the sex determination pathway, two genes that exhibit relic thermosensitive transcription in *A. mutica (Valenzuela 2008a; Valenzuela 2008b)* and *A. spinifera (Radhakrishnan et al. 2017)*. Thus, the thermal insensitivity of the *Sf1* gene in *Apalone* makes it a strong candidate for a role as sex-determining gene in *Apalone (Valenzuela 2008a; Valenzuela et al. 2006)*, perhaps as an ovarian-determining factor or a testis repressor, but this hypothesis requires further study.

Genes with conserved chromosomal location

Unlike squamates, turtle karyotypes are considered to be highly conserved (Bickham 1981; Olmo 2008). We found some evidence to support the conservation of particular gene positions but not others. One case is the mapping of *Dax1*, *Fhl2*, and *Fgf9* to a single chromosome in all cases (although these genes were affected by intrachromosomal rearrangements as discussed below). The other case identified was for *Dmrt1*, the key regulator of male sexual development, which mapped to chromosome 7 in *Glyptemys*, *Chelydra*, and *Sternotherus*; chromosome 6 in *Trachemys*, *Apalone*, *Pelodiscus*, and *Chelodina*; chromosome 5 in *Emydura*, chromosome 4 in *Pelomedusa*, and the sex chromosomes of *Staurotypus*. Our results for *Dmrt1* confirm its chromosomal location reported earlier in *Trachemys (Ferguson-Smith 2007)*, *Staurotypus (Kawagoshi et al. 2014)*, and *Pelodiscus (Matsuda et al. 2005)*. Despite the difference in the number of the chromosome to which *Dmrt1* mapped, previous studies provided evidence that some of these chromosomes (TSC6, PSI6, and STR-XY) are homologous to the chicken Z, the sex chromosome of chicken (Matsuda et al. 2005; Montiel et al. 2016a). The location of *Dmrt1* across these turtles is largely consistent with previous reports that the six largest chromosomes are relatively conserved between turtle and chicken (Matsuda et al. 2005), albeit not fully, since numerous rearrangements between turtle and chicken have been documented (Badenhorst et al. 2015). We hypothesize that EMA5 and PSU4 are likely homologous to the other chromosomes to which *Dmrt1* maps.

Genes affected by putative chromosomal fusions

Additionally, our BAC clone mapping results also revealed evidence of a likely chromosomal fusion, as *Sf1* and *Rspo1* co-localize in a single macro-chromosome in *Pelomedusa* (PSU2) and in two micro-chromosomes in all other Turtles examined here. The most parsimonious explanation given the phylogenetic relationships of these species is that a fusion event occurred such that the two ancestral micro-chromosomes harboring these two genes fused and became part of a larger chromosome in the family Pelomedusoidea. This corresponds to the long arm of PSU2, where both genes co-localize. This result supports the

hypothesis that the evolutionary reduction in diploid number within the side-neck suborder of turtles (Pleurodira), and especially in Pelomedusoidea, occurred via chromosomal fusions (Montiel et al. 2016a). Furthermore, our data also indicate that the location of *Sf1* and *Rspo1* in two different micro-chromosomes may be the ancestral condition to turtles. An alternative but less likely scenario is that two partial chromosomal regions containing *Sf1* and *Rspo1*, translocated to the long arm of PSU2.

Genes affected by intra-chromosomal rearrangements

Our FISH mapping uncovered intra-chromosomal rearrangements among turtles with similar and contrasting sex-determining mechanisms. For instance, *Dax1*, *Fhl2*, and *Fgf9* mapped to a single chromosome in all cases, yet their relative position varied among taxa and can only be explained by the occurrence of transpositions or inversions during the evolution of these lineages (Fig. 1B). These include inversions encompassing *Dax1* and *Fhl2* observed in *Chrysemys*, *Apalone*, and *Pelodiscus*; and transpositions of *Dax1* to different locations along the long arm of chromosome 1 in *Pelomedusa*, *Emydura*, and *Chelodina* (Fig. 1B). Our mapping data suggest that the ancestral gene order for these three genes could be *Dax1-Fhl2-Fgf9* from which inversions may have occurred independently in *Chrysemys* and in the Trionychidae lineage. Consistent with this idea, banding patterns of chromosomes in cryptodiran turtles indicate that chromosomal rearrangements such as centric fusions, pericentric inversions, and interchanges occurred during the diversification of cryptodiran families (Bickham 1981). Furthermore, two inversions were recently reported in *Glyptemys* sex chromosomes that could have contributed to the evolution of its derived XX/XY system *(Montiel et al. 2016b)*. However, taken together, the rearrangements involving *Dax1*, *Fhl2*, and *Fgf9* identified here were not associated with transitions in turtle sex determination, because TSD turtles (*Trachemys*, *Chelydra*, and *Sternotherus*) and GSD turtles (*Glyptemys* and *Staurotypus*) shared a similar gene order, while *Chrysemys* and *Trachemys* (both TSD), exhibit contrasting rearrangements between *Dax1* and *Fhl2*.

BAC clone probes that map to scaffolds containing genes *Fhl2*, *Dax1*, and *Fgf9* painted onto the largest chromosome in all turtles, except for *Emydura* where they painted onto the

third largest chromosome (EMA3). Based on these results and a few FISH data of BAC clones that mapped to CPI1 (Montiel *et al.*, unpublished), we hypothesize that EMA3 is homologous to the long arm of the largest chromosomes in other turtles. Other less likely alternatives are that fission and fusion events occurred between EMA1 and EMA3, or that these genes have translocated from one chromosome to the other (from EMA1 to EMA3). However, these alternatives represent less parsimonious hypotheses.

In conclusion, our findings add to the discovery of chromosomal rearrangements involving regulators of sexual development in turtles as a first step to testing the hypothesis that rearrangements underlying the evolution of diploid number trigger SDM transitions, thus explaining the observed acceleration of the rates of change in chromosome number and SDM turnover in turtles (Valenzuela and Adams 2011). We found evidence to rule out the hypothesis that changes in the relative position of most of the genes in this particular focal gene set are associated with evolutionary transitions in turtle sex determination. The one exception is the translocation of *Foxl2* from an ancestral location in a macro-autosome to the sex chromosomes of *Apalone* where *Sf1* is also linked. Yet, our phylogenetically informed comparative analysis permits ruling out this hypothesis because the evolution of GSD in *Trionychidae* predates the translocation of *Foxl2* in *Apalone*. Our findings of *Sf1* in sex chromosomes strengthen *Sf1* as a candidate for a role as sex-determining gene in this GSD turtle. Further comparative analyses using an expanded set of genes from the sexual development network is needed to inform genome evolution and its contribution to enigmatic turnovers of vertebrate sex determination.

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Statement of Ethics

All procedures were approved by the IACUC of Iowa State University.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Figure Legends

Fig. 1. Phylogenetic relationships of 12 target turtle species examined and results obtained in this study. Orange species acronyms denote TSD turtles, while purple acronyms denote GSD turtles. Stars indicate branches where transitions among SDMs occur (modified from Valenzuela & Adams, 2011). **(A)** Sex-determining mechanism (SDM), diploid number (2N), and chromosomal location of the BAC probes mapped in this study. Colored cells indicate chromosome homology based on BAC-FISH data collected for a parallel study (Montiel et al., unpublished). Chromosome number in the table correspond to the value assigned in their karyotype where chromosomes are organized by decreasing size and centromere position (Montiel et al. 2016a). Asterisk denotes previous result from Badenhorst et al. (2015) for CPI. **(B)** Chromosome painting with BAC probes for *Fgf9*, *Dax1*, and *Fhl2* showing their relative position in a single chromosome across turtles. Red band denotes *Fhl2*, yellow denotes *Dax1*, and blue denotes *Fgf9*. These BAC probes painted onto the largest chromosome pair in all turtle species, except *Emydura*. The ancestral gene order for *Fgf9*, *Dax1*, and *Fhl2* is presented in those branches where it could be confidently reconstructed by parsimony. CPI = *Chrysemys picta*, TSC = *Trachemys scripta*, CSE = *Chelydra serpentina*, GIN = *Glyptemys insculpta*, STR = *Staurotypus triporcatus*, SOD = *Sternotherus odoratus*, ASP = *Apalone spinifera*, PSI = *Pelodiscus sinensis*, PUN = *Podocnemis unifilis*, PSU = *Pelomedusa subrufa*, COB = *Chelodina oblonga*, EMA = *Emydura macquarii*.

Fig. 2. Illustration of the relative position of the genes of interest and each BAC probe used in this study.

Fig. 3. Exemplar FISH results identifying the chromosomal locations of BAC clones containing genes of interest on turtle chromosome spreads. Probes labeled with biotin-dUTP are *green* (denoted by yellow arrowheads) and with digoxigenin-dUTP are *red* (denoted by yellow arrows). **A** *Fgf9* (green) and *Dmrt1* (red) in *Trachemys*. **B** *Fhl2* (green) and *Fgf9* (red) in *Emydura*. **C** *Rspo1* (green) and *Sf1* (red) in *Pelomedusa*. **D** *Rspo1* (green) and *Sf1* (red) in *Glyptemys*. **E** *Sf1*

(green) and *Rspo1* (red) in *Pelodiscus*. **F** *Wt1* (green) and *Foxl2* (red) in *Staurotypus*. **G** *Sf1* (red) in ASP. **H** *Foxl2* (green) in *Apalone*. *Boxes* contain the enlarged W and Z sex chromosomes from *Apalone* chromosome spreads.

Fig. 4. Karyotypes of target species and the chromosomal location of BAC clones containing the gene of interest. Karyotype illustrations are based on (Montiel et al. 2016a).

Table 1. Function of target genes in turtle gonadal formation

Gene	Gene name	Functions	Source
Dax1 (*NR0B1*)	Dosage-sensitive sex reversal (DSS), adrenal hypoplasia congenital (AHC) critical region, on the X chromosome, gene 1	• Involved in mammalian ovarian differentiation • Antagonist to *Sry* in Therian mammals	1, 2
Dmrt1	Doublesex (Dsx) and mab-3 related transcription factor 1	• Key regulator of male sexual development in both invertebrates and vertebrates • Required by Sertoli and germ cells • Associated with transitions in sex determination in reptiles	1, 3, 4
Fgf9	Fibroblast growth factor 9	• Key component of testis-determining pathway • First expressed in bipotential gonads • Maintains *Sox9* expression • Inactivates *Wnt4* signaling in testis	5
Fhl2	Four and a half LIM domains 2	• Binds with *Wt1* to modulate transcription of some genes during gonadal differentiation • With *Wt1*, upregulates *Dax1* during ovarian development	6
Foxl2	Forkhead box L2	• Maintains ovarian development and differentiation • Inhibits *Sox9* and *Dmrt1* expression, suppress testicular differentiation • Candidate sex-determining gene in GSD turtles	7
Gata4	GATA binding protein 4	• Candidate gene for thermosensitive testicular differentiation in TSD turtles • Sex-linked (Y_1 and X_2) in platypus	8, 9
Rspo1	R-spondin 1	• Along *Wnt4*, required for gonadal cell proliferation in both sexes before sex determination	10, 11

		• Essential for ovarian development and differentiation	
Sf1 (*AD4BP*) (*NR5A1*)	Steroidogenic factor 1	• Key regulator of steroid enzymes • Helps form genital ridges in mammals • Involved in vertebrate sexual differentiation • Candidate TSD master gene in turtles • Candidate sex-determining gene in GSD turtles	2, 12, 13
Sox9	SRY (sex determining region Y)-box 9	• Activated by *Sry*, tips the bipotential gonad towards the male fate • Associated with Sertoli cell proliferation, somatic cell expansion, migration and differentiation during testis development	2, 5, 9
Wt1	Wilms tumor protein 1	• Helps form genital ridges in mammals • Regulator of bipotential gonad formation • Candidate TSD master gene in turtles	2, 12, 14

1. (Ferguson-Smith 2007) **2.** (Ramkissoon and Goodfellow 1996) **3.** (Haag and Doty 2005) **4. (**Janes et al. 2014) **5. (Kim et al. 2006) 6.** (Du et al. 2002) **7.** (Uhlenhaut et al. 2009) **8.** (Grafodatskaya et al. 2007) **9.** (Radhakrishnan et al. 2017) **10.** (Chassot et al. 2012) **11.** (Tomaselli et al. 2011) **12.** (Valenzuela 2008b) **13.** (Valenzuela et al. 2006) **14.** (Valenzuela 2008a)

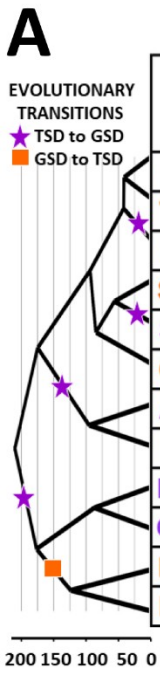
Table 2. Chromosome locations of target genes in *Chrysemys picta* (CPI), human and chicken [from (Badenhorst et al. 2015) or this study (denoted by ¤)]

Gene name	BAC ID	CPI genome scaffold *	Chromosome location in CPI	Chromosome location in chicken	Chromosome location in human
Fhl2	*61H12*	NC_024218.1	1q	1	2
Dax1	**147L13**	NC_024218.1	1q	1	**X**
Fgf9	**55A6**	NC_024218.1	1q	1	13
Sox9	**337P6**	NC_024220.1	3q	18	17
Wt1	*88H12*[§] 64G12 147H2	NW_007281759.1	4q	5	11
Dmrt1	**41L5 44L23**	NC_024223.1	7	**Z**	9
Rspo1	*121H12*	NC_024231.1	18	23	1
Sf1	**66P24 225M10**	NW_007281439.1	Unplaced scaffold	17	9
Foxl2	*380M2*	NW_007359912.1	Unplaced scaffold	9	3
Gata4	*225G19*	NW_007281425.1	3q[¤]	3	8

* = CPI assembly from NCBI Chrysemys_picta_bellii-3.0.3. § = BAC originally mapped in CPI (Badenhorst et al. 2015) but enriched in repeats which precluded clean hybridization in some more distant turtles. **BAC IDs in bold font** denote fully sequenced BAC clones containing gene of interest; underlined BAC IDs denote BAC clones that were screened by PCR using gene-specific primers, Sanger-sequenced, and validated as containing the gene of interest; and *BAC IDs in italics* denote fully sequenced BAC clones that mapped to a scaffold in the painted turtle genome assembly containing the gene of interest.

Figure 1

A



Gene	*Fhl2*	*Dax1*	*Fgf9*	*Sox9*	*Gata4*	*Wt1*	*Dmrt1*	*Foxl2*	*Sf1*	*Rspo1*
BAC ID	61H12	147L13	55A6	337P6	225G19	88H12 64G12 147H2	41L5 44L23	380M2	66P24 225M10	121H12
2N										
CPI	50	1*	1*	1*	3*	3	4*	7*	9	20	18*
TSC	50	1	1	1	3	3	4	6	9	18	23
GIN	50	1	1	1	3	3	4 (XY)	7	9	17	19
SOD	56	1	1	1	3	3	5	7	9	19	22
STR	54	1	1	1	3	3	5	4 (XY)	9	17	19
CSE	52	1	1	1	3	3	6	7	9	16	19
ASP	66	1	1	1	3	3		6	zw	zw	26
PSI	66	1	1	1	3		4	6		21	24
EMA	50	3	3	3	1q	1q	1p	5	7	18	20
COB	54	1	1	1	3	3	5	6	9	20	14
PUN	28			1							
PSU	36	1	1	1				4		2	2

B

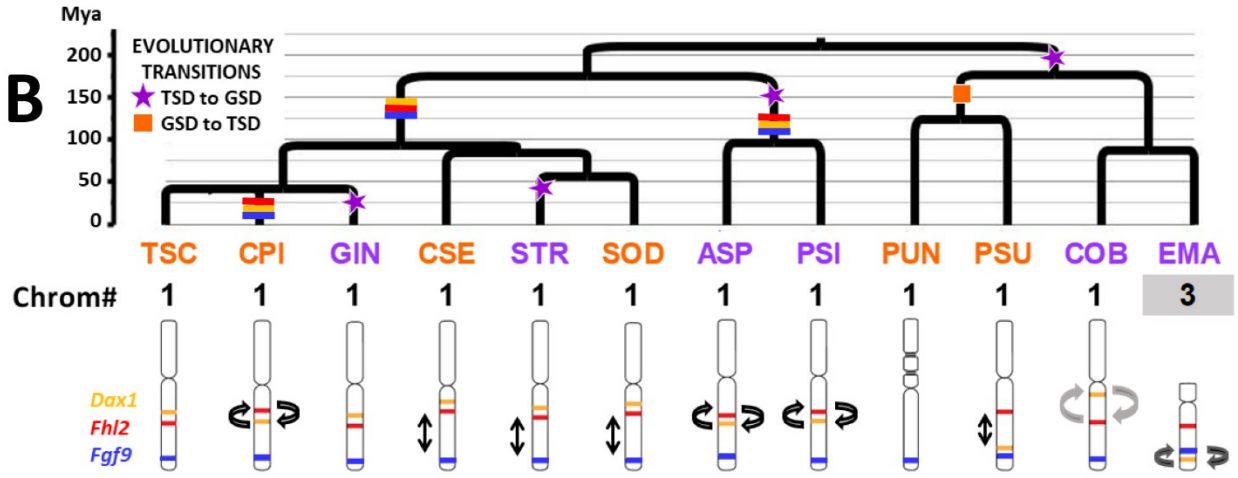
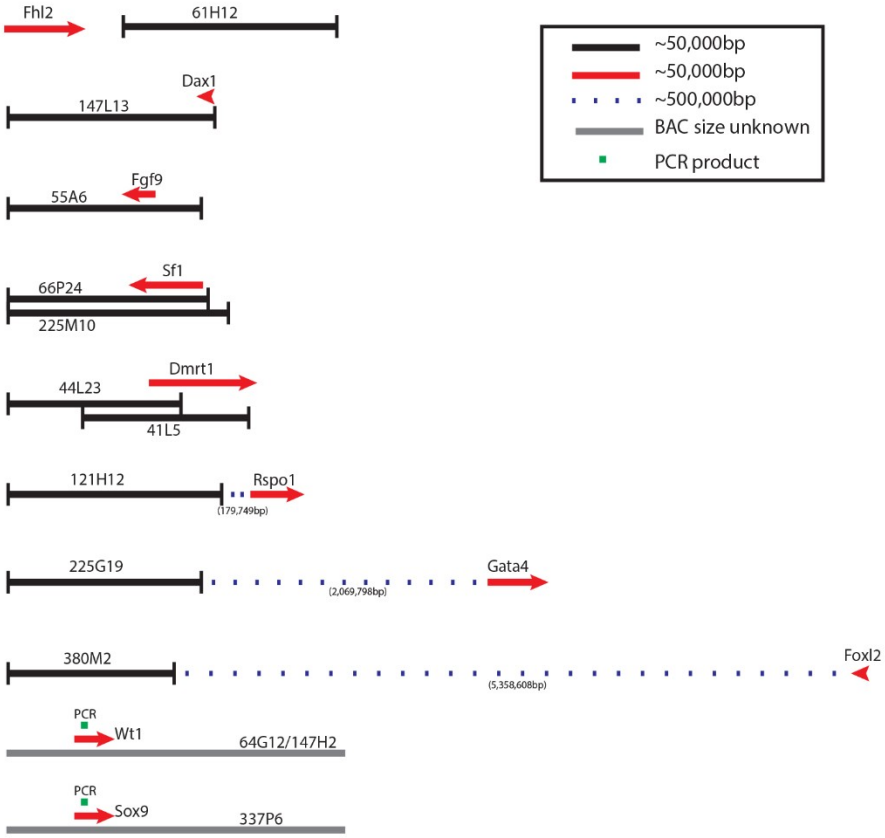


Figure 2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Dax1
147L13
Fgf9
55A6
Sf1
66P24
225M10
Dmrt1
44L23
41L5
121H12
Rspo1
(179,749bp)
225G19
Gata4
(2,069,798bp)
380M2
Foxl2
(5,358,608bp)
PCR
Wt1
64G12/147H2
PCR
Sox9
337P6
~50,000bp
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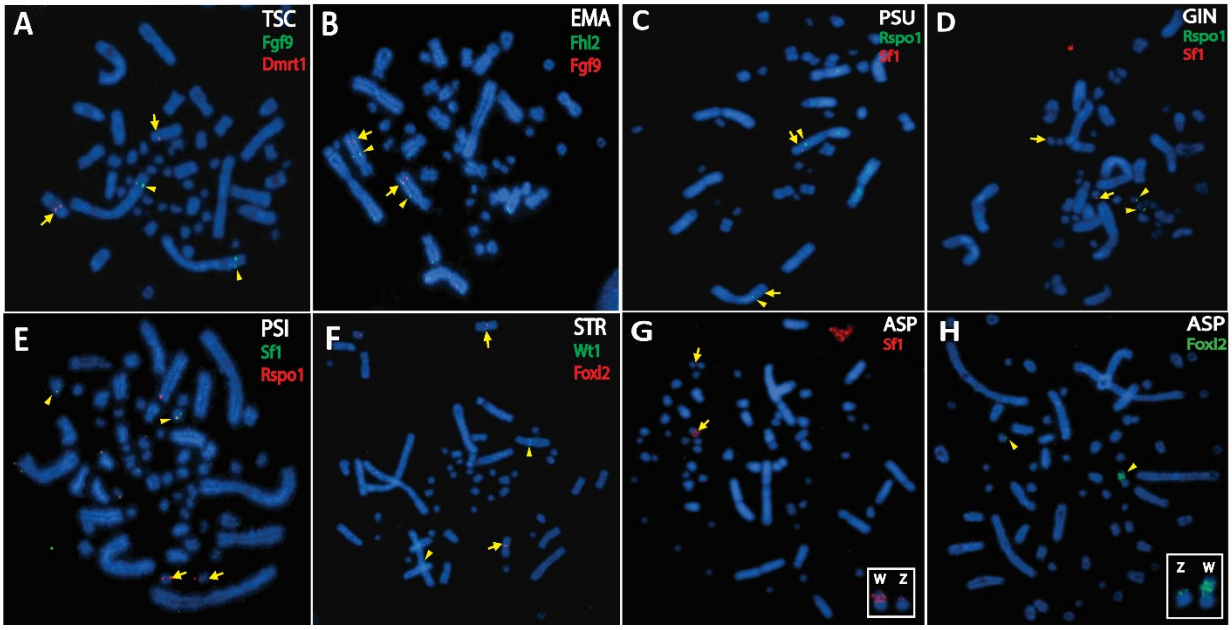
~500,000bp
BAC size unknown
PCR product

Figure 3


A
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E
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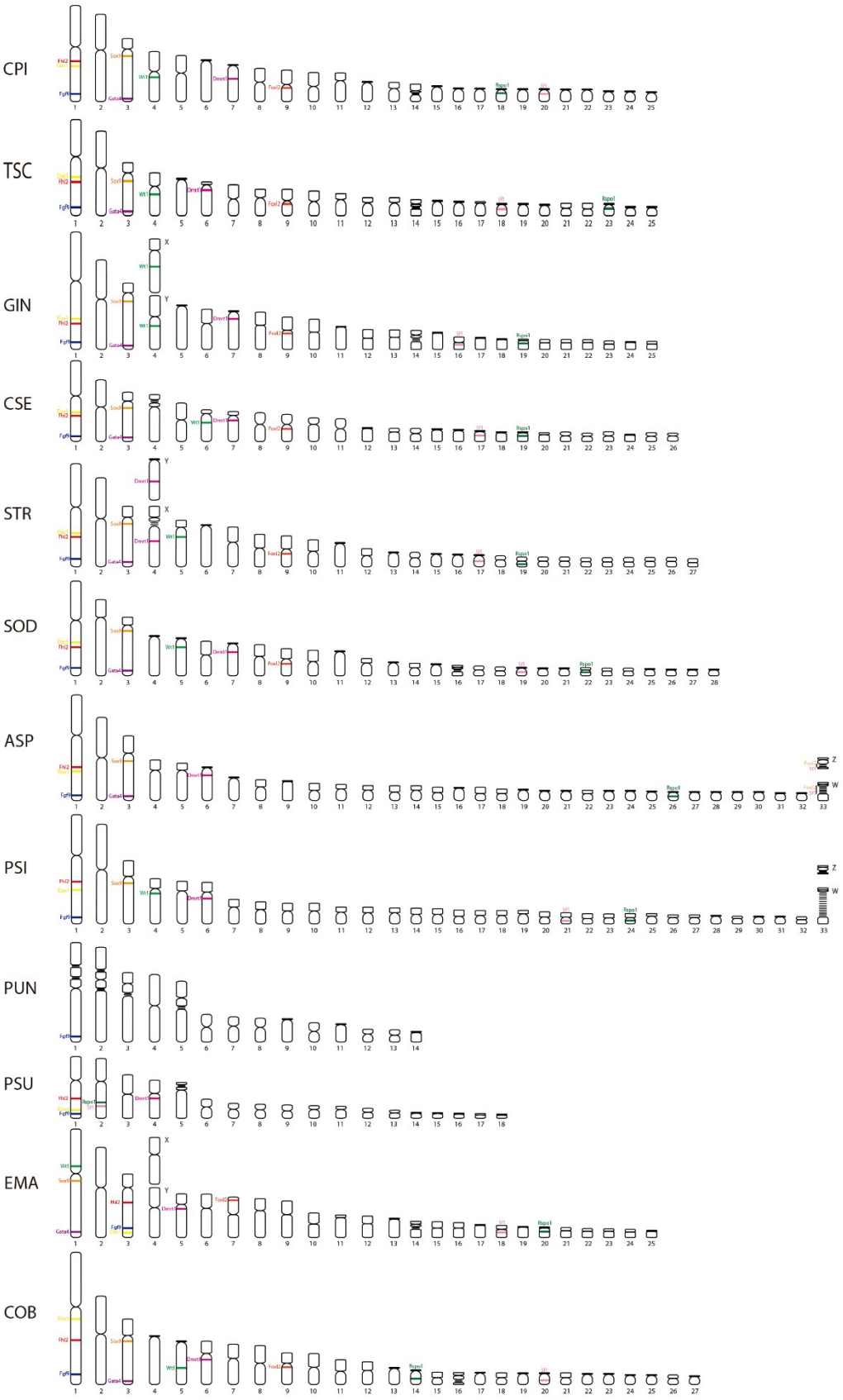
Figure 4


CPI
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